

# Morphogenesis and Organogenesis in the Regenerating Planktotrophic Larvae of Asteroids and Echinoids

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**Abstract.** In a previous study, we described complete body regeneration (with organogenesis) following surgical bisection in the planktotrophic larvae of the asteroids *Luidia foliolata* and *Pisaster ochraceus*. Here we present further detailed observations of these unique regenerative processes not presented in the previous paper. Furthermore, we describe for the first time complete regeneration following surgical bisection of planktotrophic larvae of the regular echinoid *Lytechinus variegatus* and the irregular echinoid *Dendraster excentricus*. Larvae of both asteroids and echinoids displayed a capacity for rapid regeneration regardless of their developmental stage. Within 48 h after bisection, aggregations of mesenchyme cells with pseudopodia were observed at the site of surgical bisection. These cellular aggregations were similar in appearance to the mesenchymal blastemas that form in adult echinoderms prior to their arm regeneration, and to those described in other deuterostomes that undergo regeneration. When asteroid larvae were surgically bisected in the early stages of their development, clusters of mesenchyme cells developed into completely new pairs of coelomic pouches located anterior to the newly regenerated digestive tract. This indicates that cell fate in regenerating asteroid larvae remains indeterminate during early development. In the larvae of *P. ochraceus*, regardless of the developmental stage at the time of bisection, both the anterior and posterior portions regenerated all their missing organs and tissues. However, the larvae of *L. foliolata* displayed differential regenerative capacity in bisected larval halves at the late bipinnaria stage. The differences observed may be due to differences in larval development (*L. foliolata* has no brachiolaria stage), and may have evolutionary implications. In the regular echinoid *L.*

*variegatus*, both larval portions regenerated into morphologically and functionally normal larvae that were indistinguishable from non-bisected control larvae. The regenerative processes were similar to those we observed in planktotrophic asteroid larvae. Regenerating larvae readily metamorphosed into normal juveniles. In the irregular echinoid *D. excentricus*, posterior portions of larvae completed regeneration and metamorphosis, but anterior portions regenerated only partially during the 2-week study. Our observations confirm that asteroid and echinoid larvae provide excellent models for studies of regeneration in deuterostomes.

## Introduction

Regeneration has been described at both the cellular and the tissue level in many animals including planarians, crustaceans, reptiles, and amphibians (Goss, 1969; Mattson, 1976; Bagnà *et al.*, 1989; Martin, 1997; Hopkins, 2001). Many adult echinoderms, including asteroids, ophiuroids, and holothuroids, are known to possess considerable regenerative capacities as well as the ability to reproduce by clonal division (Emson and Wilkie, 1980; Mladenov and Burke, 1994). The occurrence of cloning in planktotrophic larvae of asteroids and ophiuroids has also been reported (Bosch, 1988; Bosch *et al.*, 1989; Rao *et al.*, 1993; Jaeckle, 1994; Balser, 1998). However, complete regeneration in echinoderm larvae after surgical bisection has only recently been documented. Bipinnaria and brachiolaria larvae of the asteroids *Luidia foliolata* and *Pisaster ochraceus* were surgically bisected into anterior and posterior portions, and the regeneration process was followed for 2 weeks (Vickery and McClintock, 1998). Both portions of the larvae in both species completely regenerated all missing organs and tissues.

The discovery of regeneration in planktotrophic asteroid

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larvae provided a previously unknown model system for the molecular study of regeneration in deuterostomes (Vickery, 2001; Vickery *et al.*, 2001a, b). This model offers several advantages. For example, echinoderms share many developmental traits with other deuterostomes, including vertebrates (Halanych and Passamaneck, 2001; Nielsen, 2001). Also, asteroid larvae possess simple body structures compared to adults, and their transparent bodies make it possible to observe the regeneration process not only externally, but also internally.

In the present study we used larvae of the asteroids *Luidia foliolata* and *Pisaster ochraceus* to gain detailed information about the regeneration process at the cellular and organ levels. We examined the regenerative capacity and organogenesis of larvae of both asteroid species throughout all developmental stages. This study provides the first assessment of the ability of discrete stages of larvae to regenerate. We also report on the regenerative capacity of surgically removed portions of the larval body in *Pisaster ochraceus*.

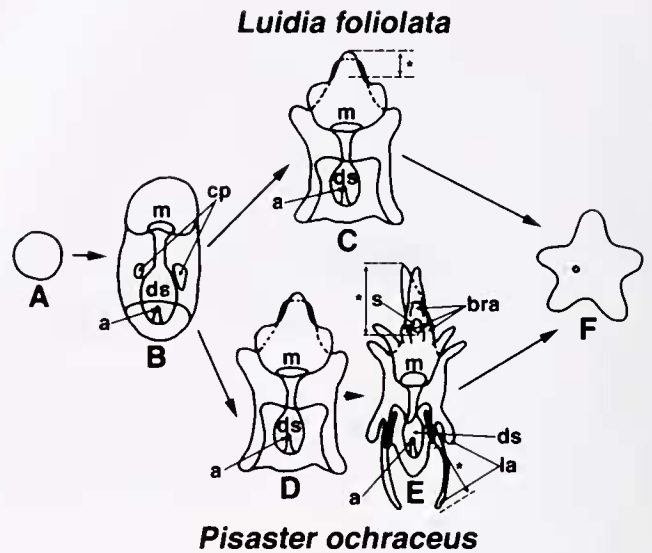
Although early studies examined the ability of echinoid larvae to regenerate specific missing tissues (reviewed in Hörstadius, 1973, and Vickery *et al.*, 2001b), few studies similar to those we have outlined above for planktotrophic asteroid larvae have examined regenerative characteristics in planktotrophic echinoid larvae (Mortensen, 1921, 1938; Czihak, 1965). Given our immense knowledge of aspects of echinoid molecular genetics and cellular development, echinoid larvae could become a model system for studies of deuterostome regeneration. In the present study we examine the regenerative capacity of both the planktotrophic pluteus larvae of the regular echinoid *Lytechinus variegatus* and the irregular echinoid *Dendraster excentricus*.

## Materials and Methods

### Asteroids

**Larval cultures.** Sexually mature individuals of *Luidia foliolata* and *Pisaster ochraceus* were collected near San Juan Island, Washington, in the late spring to early summer of 1998. Artificial fertilizations were conducted by intracoelomic injection of 1-methyladenine (Kanatani, 1969), and ambient seawater temperature (12–15 °C) was maintained during the experiments. After the embryos established functional digestive systems, larvae were fed a quantitatively equal mixture of the single-celled algae *Chaetoceros calcitrans*, *Dunaliella tertiolecta*, and *Isocrysis galbana*. Standard protocols for larval culturing were followed (Strathmann, 1987).

First we investigated larval regenerative capacities in three representative stages of larval development, chosen on the basis of larval anatomy. Early-stage larvae were defined, for both species, as embryos (Fig. 1A) that had developed into early bipinnaria larvae with a functional digestive system and a pair of non-connected coelomic pouches (Fig.



**Figure 1.** Diagram of two types of planktotrophic larval asteroid development. The embryo (A) develops into a planktotrophic bipinnaria larva (B). Bipinnaria larvae of *Luidia foliolata* develop into a late bipinnaria (C), and complete metamorphosis to become juvenile asteroids (F) while swimming in the water column. Bipinnaria larvae of *Pisaster ochraceus* (D) develop into brachiolaria (E), after which they complete metamorphosis to the juvenile stage (F). Arrows with \* in (C) and (E) show area of larva removed to examine regeneration of partial body parts. a, anus; bra, brachiolar apparatus; cp, coelomic pouches; ds, digestive system; la, larval arms; m, mouth; s, sucker.

1B). Mid-stage larvae were defined as fully grown bipinnaria larvae in *L. foliolata* (Fig. 1C), and as either fully developed bipinnaria (Fig. 1D) or brachiolaria (Fig. 1E) larvae in *P. ochraceus*. All mid-stage larvae possessed long larval arms, and *P. ochraceus* brachiolaria larvae had a brachiolar apparatus in the anterior region. Internally, the left and right coelomic pouches were connected at the anterior ends of the larvae; in brachiolaria larvae, the brachiolar apparatus possessed an underlying coelom. Late-stage larvae were defined as either bipinnaria or brachiolaria larvae with an adult rudiment on the posterior portion. The coelom had developed hydrocoelic lobes that would eventually form the water vascular system in the juvenile (Fig. 1F). Moreover, spicules were present that would eventually form the juvenile skeletal system.

**Surgical bisection.** At each discrete larval stage, subsamples of larvae ( $n = 20$ ) of both species were first measured, then surgically bisected with a scalpel across the horizontal larval axis, at a point equidistant between the anterior and posterior poles (Vickery and McClintock, 1998). Bisected anterior and posterior portions and non-bisected control larvae ( $n = 20$ ) for each stage were maintained separately for 2 weeks in fingerbowls placed in a water table with a circulating seawater system. The seawater in each fingerbowl was changed every 3 days (Strathmann, 1987). Using dissecting and compound light micros-

copy, we examined and measured the specimens daily. Growth was determined by their increase in length. A Nikon Optiphot-2 compound microscope was used to photograph larvae. All measurements were analyzed statistically, and a value of  $P < 0.05$  was employed to assess statistical significance.

To further assess larval regenerative capacity, we examined *P. ochraceus* because this species possesses both bipinnaria and brachiolaria larval stages. The anterior tip containing the axocoel was removed from both mid-stage bipinnaria ( $n = 20$ ) and brachiolaria ( $n = 20$ ) larvae (including the brachiolar apparatus) (Fig. 1C, D). Moreover, sets of posterior larval arms were removed from brachiolaria larvae ( $n = 20$ ) (Fig. 1C, D). The dissected fragments and the larvae from which they were removed were maintained separately and observed for 2 weeks as described above.

### Echinoids

**Larval cultures.** Adult specimens of *Lytechinus variegatus* were collected from shallow waters (about 0.3–2 m depth) from the Gulf of Mexico near Port Saint Joseph, Florida, during late spring of 1999. Individuals were maintained in aerated, 160-l recirculating aquaria containing artificial seawater (ASW) (Instant Ocean, Aquarium Systems, Mentor, OH) and held at about 20–25 °C. Larvae were obtained from a single male-female pair by artificial fertilization using gametes from spawn triggered via intracoelomic injection of 0.5 M KCl. Embryological and larval development were monitored and photographed using an Olympus CO-11 binocular dissecting microscope and an Olympus CH binocular compound light microscope equipped with a camera. Cultures were maintained with gentle stirring at about 20–23 °C at a density of no greater than 0.8 larvae/ml, and the culture water was changed every 3 days. Larvae were fed approximately  $5 \times 10^4$  cells/ml of a diet composed of equal amounts of the single-celled algae *Chaetoceros calcitrans*, *Dunaliella tertiolecta*, and *Isocrysis galbana* (Strathmann, 1987). Thirteen days after fertilization, the larvae had developed into early 8-armed pluteii. These larvae were cultured for an additional 2 days to allow them to attain the late 8-armed pluteus stage for use in the present experiment.

The 8-armed pluteus larvae of the irregular echinoid *Dendraster excentricus* were cultured at Friday Harbor Laboratories, University of Washington, during the summer of 1998. These larvae, which were obtained by artificial fertilization using methods similar to those described above, were cultured in natural seawater in glass fingerbowls (250 ml). The fingerbowls were held in a circulating seawater system at 12–15 °C. Larvae were fed the single-celled algae *Rhodomonas* sp. (Strathmann, 1987). Larval regeneration was monitored and photographed using a Nikon Wild M-5

dissecting microscope and a Nikon Optiphot-2 microscope equipped with a camera system. Only 15-day-old larvae were used in the present experiment.

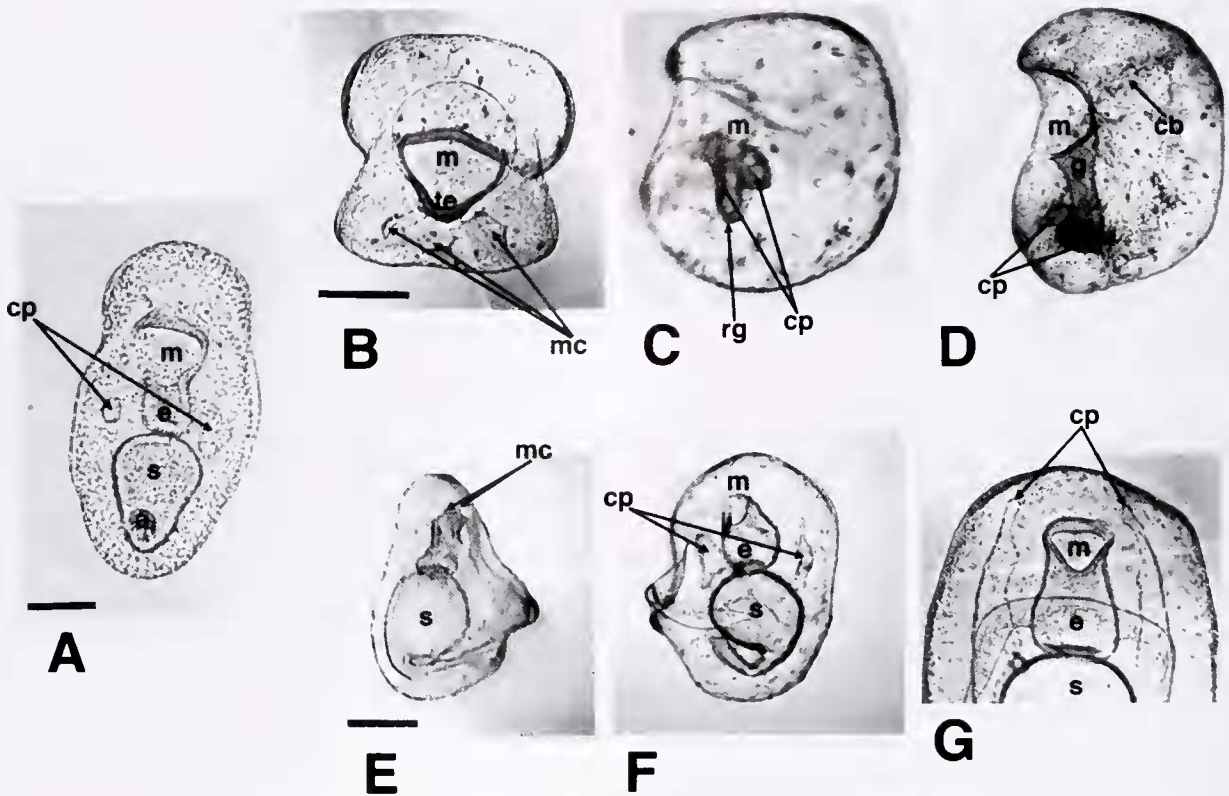
**Surgical bisection.** Pluteus larvae of the regular echinoid *L. variegatus* were surgically bisected into anterior and posterior sections at a point about equidistant between the anterior and posterior poles (Figs. 9A, 10A) (Vickery and McClintock, 1998; Vickery *et al.*, 2001b; Vickery, 2002). Before bisection, the larvae ranged from about 600 to 1100  $\mu\text{m}$  in length (along the anterior to posterior larval axis), possessed a larval skeleton (Fig. 9A), and were actively swimming and feeding. The same procedure was used to surgically bisect pluteus larvae of the irregular echinoid *D. excentricus*. Larvae ranged from about 700 to 1000  $\mu\text{m}$  in length (along the anterior to posterior larval axis) and like the larvae of *L. variegatus*, possessed a larval skeleton before they were bisected (Fig. 10A). All anterior and posterior portions of larvae were maintained in 250-ml glass fingerbowls under the culture conditions described above for each species. Non-bisected control larvae were simultaneously cultured under identical conditions for comparison. Anterior and posterior larval portions of both species were observed and photographed daily for 14 days.

## Results

### Regeneration in larval asteroids

**Regeneration in early-stage larvae.** Early-stage larvae of *Luiddia foliolata* were surgically bisected 21 days after fertilization. Before bisection the bipinnaria larvae measured  $710 \pm 80 \mu\text{m}$  in length ( $n = 20$ , Fig. 2A). After bisection, both the anterior and the posterior portions of the larvae continued utilizing their ciliary bands to swim and collected phytoplankton to feed. Even though the anterior portions had lost the gut due to surgical bisection, phytoplankton was still accumulating in the oral cavity. The posterior portions of the larvae collected phytoplankton directly through the esophagus. Within 24 h, rough edges of tissues originally seen at the site of bisection in both the anterior and posterior portions of the larvae had become smooth. Within 48 h of bisection, mesenchyme cells with pseudopodia appeared at the site of bisection (Fig. 2B, E) in both the anterior and posterior larval portions. In the anterior portions, a thickening of the former upper esophagus/lower mouth region became evident (Fig. 2B); and aggregations of mesenchyme cells around this thickened area became more prominent. Eventually this thickened wall invaginated into the body cavity, forming a tube (Fig. 2C). Similar aggregations of mesenchyme cells were observed in the bisected posterior portions at various locations throughout the regenerating larval body, most notably at the site of bisection.

The anterior portions of bisected larvae gradually decreased in size after bisection (immediately after bisection =  $306 \pm 47 \mu\text{m}$  in length; 11 days later =  $233 \pm 29$

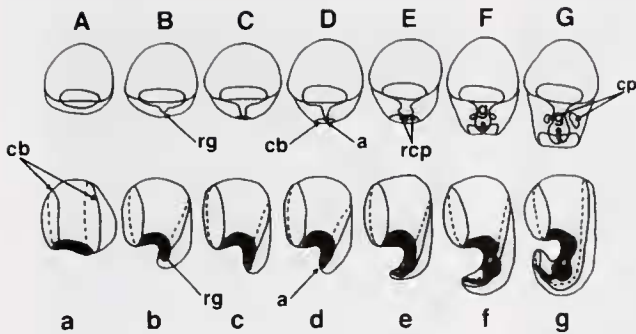


**Figure 2.** Light photomicrographs of regenerating early-stage bipinnaria larvae of *Luidia foliolata*. (A) A 21-day-old bipinnaria larva with a pair of coelomic pouches located on the sides of the larval esophagus. Two days after bisection, mesenchyme cells are apparent at the surgical plane in the anterior (B) and posterior (E) portions. Note that the anterior portion (B) does not possess any coelomic components following surgical bisection and that a thickening of the epidermis is evident at the rear part of the former mouth. Three days after bisection, the larval epidermis invaginates into the body cavity and forms a new pair of coeloms evaginating from the upper portion of the newly formed rudimentary gut in the anterior portion (C). The posterior portion (F) increases in size, and regeneration of the missing mouth and elongation of the right and left coeloms are evident (F). Seven days after bisection, the newly formed coeloms are clearly separated from the newly formed gut in the anterior portion (D), further extension of the right and left coelomic pouches is evident, and the lost mouth has completely regenerated (G). Scale bars = 200  $\mu\text{m}$  in (A), 100  $\mu\text{m}$  in (B)–(D), 100  $\mu\text{m}$  in (E)–(F). a, anus; cb, ciliary band; cp, coelomic pouches; e, esophagus; g, gut; m, mouth; mc, mesenchyme cell; rg, rudimentary gut; s, stomach.

$\mu\text{m}$  in length;  $n = 20 \pm 1$  SD). However, the posterior portions of the bisected larvae, which retained a functional digestive system and continued feeding throughout the regeneration process, continued to grow (immediately after bisection =  $378 \pm 31$   $\mu\text{m}$  in length; 11 days later =  $495 \pm 136$   $\mu\text{m}$  in length;  $n = 20 \pm 1$  SD). Seven days after bisection (28 days after fertilization), the aggregation of mesenchyme cells (described above) seen in the anterior portions of the larvae appeared to be involved in development of a new pair of coelomic pouches that evaginated from the anterior portion of the newly formed "digestive tube" (Fig. 2C). The digestive tube then elongated towards the posterior and reconnected to the posterior portion of the larval wall, forming an opening that eventually became a new anus (Fig. 2D). By 7 days after bisection, the posterior portions of the bisected larvae had almost completely re-

generated the mouth (Fig. 2F, G), and an elongation of the coelomic pouches was prominent. Once the anterior portions of the bisected larvae had reformed a complete digestive tube, actual feeding and digestion of phytoplankton began; differentiation of the digestive tube into a new esophagus, stomach, and intestine was observed by day 10 (Fig. 2D). After about 12–14 days, both the anterior and posterior portions of the bisected larvae had completely regenerated all missing body structures, including all internal organs.

From our observations, we constructed a schematic diagram of regeneration and organogenesis of the anterior portion of bisected asteroid larvae (Fig. 3). Immediately after bisection, the anterior portion is left with no coelom components (Fig. 3Aa), but it retains one complete preoral ciliary band and one incomplete postoral ciliary band. To



**Figure 3.** Diagrams of organogenesis in early-stage anterior portions of surgically bisected regenerating bipinnaria larvae. (A) through (G) represent a frontal view, (a) through (g) represent a lateral view. (A) and (a) represent the anterior portion immediately after surgical bisection. (B) and (b) through (D) and (d) represent the formation of a new gut described in Fig. 2(B) and (C). (E) and (e) through (G) and (g) represent the formation of a new set of coeloms and differentiation of the newly formed gut into esophagus, stomach, and intestine described in Fig. 2 (D). a, anus; cb, ciliary band; cp, coelomic pouch; g, gut; rcp, rudiment of coelomic pouch; rg, regenerating gut.

regenerate the missing digestive system, the body wall begins to invaginate (Fig. 3Bb, Cc), eventually forming a new anus (Fig. 3Dd). During this time, the postoral ciliary band extends toward the posterior. Meanwhile, from the anterior portion of the new gut, an entirely new set of coeloms is formed by lateral evagination of the newly formed digestive system (Fig. 3Ee), which then separate from the gut and elongate anteriorly and posteriorly alongside the newly formed digestive system (Fig. 3Ff).

Afterwards, complete differentiation of the newly formed digestive system, completion of regeneration of the lost components of the postoral ciliary band, and further development of both coeloms are observed (Fig. 3Gg). Our observations of the regeneration process in early-stage bipinnaria larva of *P. ochraceus* were virtually identical to those described above for *L. foliolata*.

**Regeneration in mid-stage larvae.** Regenerative processes in bisected mid-stage larvae of both *L. foliolata* and *P. ochraceus* were virtually identical to those described above for early bipinnaria larva of *L. foliolata* (as previously reported by Vickery and McClintock, 1998). The only difference was that the anterior portions of mid-stage bisected larvae contained an axocoel, and therefore did not have to regenerate the coelomic pouches as they were already present. Bisected anterior portions continued to swim and collect phytoplankton in the oral cavity (Fig. 4A). An invagination of the body wall occurred after the appearance of mesenchyme cells, and the coelomic pouches elongated toward the posterior (Fig. 4B). A new digestive system completed regeneration about one week after bisection (Fig. 4C). The posterior portions, which retained a functional gut, continued to digest phytoplankton (Fig. 4D). During the

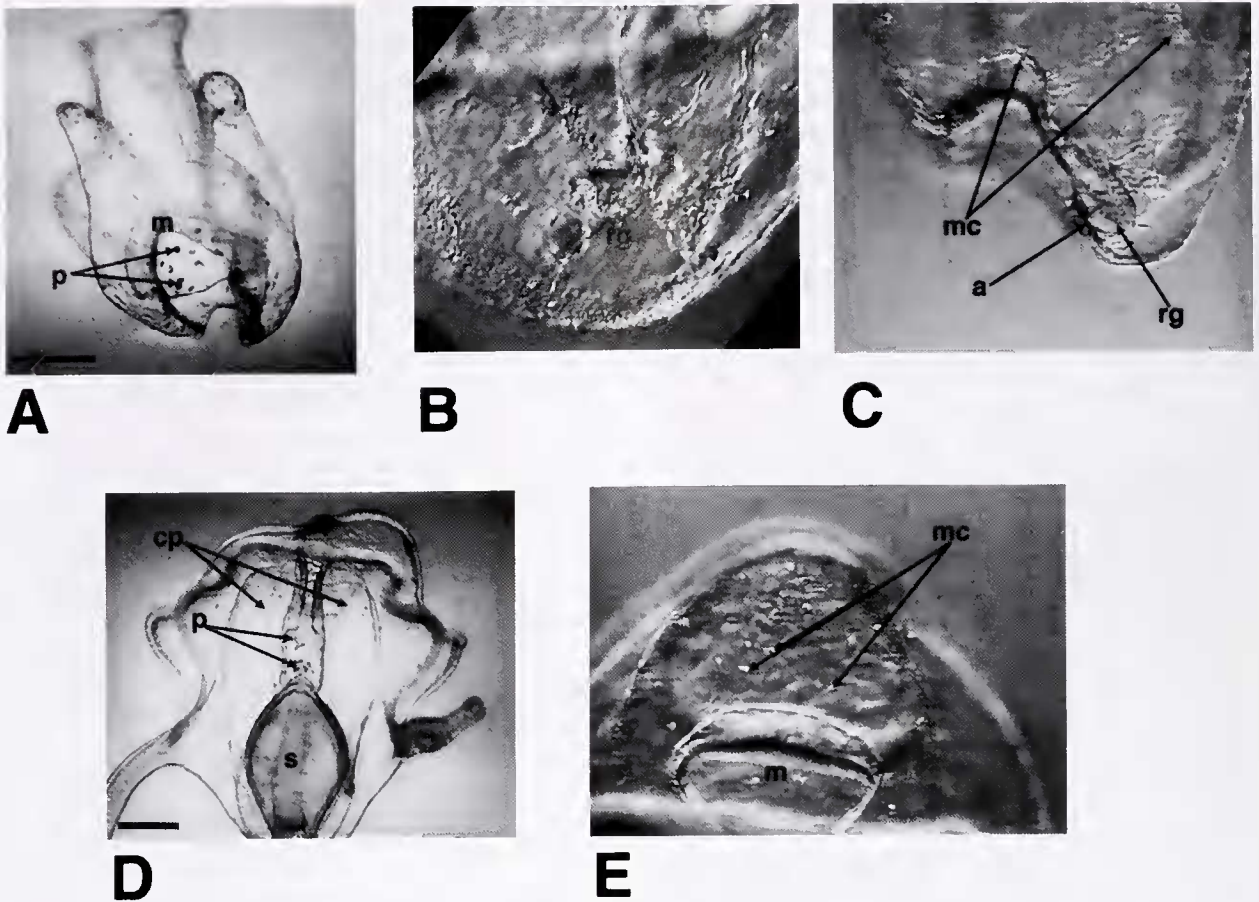
process of regeneration of the lost anterior portions, mesenchyme cells with pseudopodia were evident (Fig. 4E).

**Regeneration in late-stage larvae.** In *L. foliolata*, the anterior portions of bisected larvae underwent regeneration as described above for mid-stage bipinnaria larva ( $n = 30$ , Fig. 5A). However, the posterior portions of bisected larvae, each possessing a juvenile rudiment, invariably metamorphosed into juveniles within 3 days ( $n = 30$ , Fig. 5B) (Vickery and McClintock, 1998). The resulting juveniles measured about 500  $\mu\text{m}$  in diameter, which was not statistically different ( $P > 0.05$ ) than the size of control juveniles ( $n = 20$ ) from non-bisected larvae.

In contrast, both the anterior and posterior portions of late-stage larvae of *P. ochraceus* underwent complete regeneration, without exception, and with no mortality ( $n = 350$ , Fig. 5C, D), in a process similar to that described above for mid-stage larvae. However, formation of a new juvenile (adult rudiment, including the spicules and hydrolobes) in association with the anterior portions of bisected larvae occurred at an accelerated rate (within only 7 days). In comparison, the control larvae required an additional 7–10 days (14–21 days total) to form an equivalent adult rudiment on the posterior portions of the larvae. Within 7 days of late-stage bisection, the anterior portions of bisected larvae were morphologically and functionally indistinguishable from control larvae, and later metamorphosed into juveniles that were indistinguishable both morphologically and functionally from juveniles obtained from non-bisected control larvae.

During the regeneration process of the posterior portions of bisected larvae, a new larval body formed in each posterior portion (Fig. 5E). The newly formed secondary larval body first developed into a bipinnaria (Fig. 5F), and later formed a brachiolar apparatus, without exception (Fig. 5G). After completing regeneration of the brachiolar apparatus, the posterior portions metamorphosed into juveniles that were morphologically and functionally indistinguishable from juveniles resulting from non-bisected control larvae. The juveniles resulting from metamorphosis of regenerated larvae (both anterior and posterior portions) measured about 500  $\mu\text{m}$  in diameter ( $n > 20$ ), a size not statistically different ( $P > 0.05$ ) than that seen in control juveniles ( $n = 20$ ) from non-bisected larvae.

**Regeneration of partial larval body parts in *Pisaster ochraceus*.** The far ends of the anterior tips were removed from both bipinnaria and brachiolaria larvae of *P. ochraceus* ( $n = 10$  each). Immediately after removal from bipinnaria larvae, the anterior tips, which contained both the axocoel and portions of the preoral and postoral ciliary bands, continued to swim (Fig. 6A). After 2 weeks the anterior tips showed no signs of regeneration; but they retained their ability to swim, and they displayed muscle contractions (Fig. 6B). In contrast, the posterior portions of the larvae partially regenerated (Fig. 6C) and proceeded to become



**Figure 4.** Light photomicrographs of regenerating mid-stage larva of *Pisaster ochraceus*. (A) through (C) represent an anterior larval portion and (D) and (E) represent a posterior larval portion. The anterior portion continues to swim in a fashion similar to the non-bisected control larvae, and collects phytoplankton into the oral cavity using the ciliary bands (A), even though it lost its digestive system during the surgical bisection. (B) shows invagination of the thickened rear part of the former mouth into the body cavity to form a rudimental digestive tube, which later becomes a new digestive system. A lateral view of the anterior portion (C) shows how the rudimental gut forms an external opening, forming a new anus. Note that many mesenchyme cells with pseudopodia are present. The posterior portion (D) continues to swim and feed, and increases in size even during regeneration. (E) shows the newly-regenerated anterior portion containing mesenchyme cells with pseudopodia. Scale bars = 200  $\mu\text{m}$  in (A) and (D). a, anus; cp, coelomic pouches; m, mouth; mc, mesenchyme cell; p, phytoplankton; rg, rudimental gut.

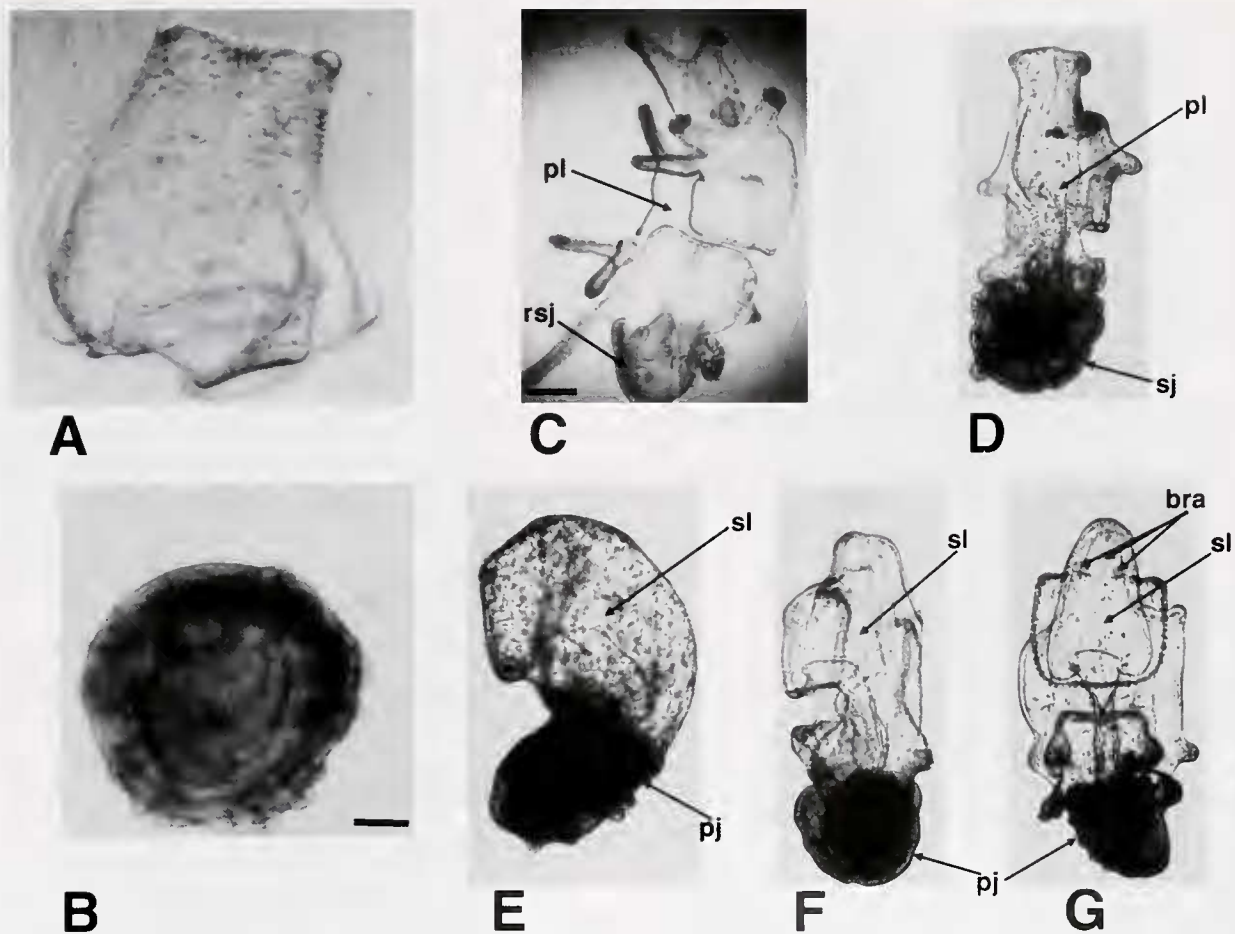
brachiolaria larvae. When the tips were removed from brachiolaria larvae, the tips, which retained the brachiolar apparatus, continued to swim using cilia (Fig. 7A), but no signs of regeneration of the larval body were evident after 2 weeks (Fig. 7B). However, the posterior portions regenerated the lost brachiolar apparatus within 7 days, as shown in Figure 7C.

Regeneration of short, stubby arm buds was observed in many brachiolaria larvae when the larval arms were surgically removed (Fig. 8A). In most cases, larval arms regenerated but never reached the same length as before. Sometimes no regeneration of the larval arms occurred at all. The severed larval arms continued to swim in a spiral fashion

(Fig. 8B), and muscular contractions could still be observed in the still-swimming severed arms after one week (Fig. 8C). After 2 weeks, no regeneration was observed in the severed arms; however, the arms themselves continued to swim actively with no mortality (Fig. 8D).

#### *Regeneration in larval echinoids*

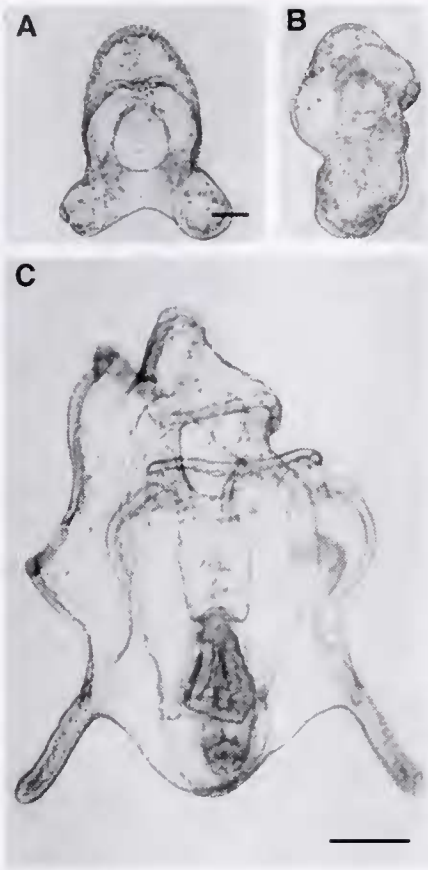
Within 5 days, anterior and posterior portions of pluteus larvae of the regular echinoid *L. variegatus* regenerated missing body components including a digestive system and a larval mouth, respectively (Fig. 9 A-E). The morphogenetic and organogenic processes appeared to be similar to



**Figure 5.** Three days after bisection of a late-stage larva of *Luidia foliolata*, the anterior portion is shown in the process of regeneration (A). In contrast, the posterior portion completes metamorphosis and becomes a juvenile (adult) (B), as we previously reported (Vickery and McClintock, 1998). After 3 days, in late-stage larvae of *Pisaster ochraceus*, the anterior portion has partially regenerated the lost primary juvenile (adult) rudiment, forming a secondary juvenile (adult) rudiment on the posterior portion (C). About 7 days after bisection, a lateral view of the anterior larva (D) shows complete regeneration of a secondary juvenile (adult) rudiment, during which the larval arms are absorbed, suggesting a progression of the process of metamorphosis. Three days after bisection, a secondary larval body is in the process of regenerating from the bisected primary juvenile (adult) rudiment (E). The secondary larval body then becomes a seemingly normal late-stage bipinnaria larva (with juvenile rudiment) after 7 days (F). By about 9 days after bisection, the secondary larval body (with juvenile rudiment) has regenerated the lost brachiolar apparatus (G). Scale bars = 200  $\mu\text{m}$  in (A)–(B), 100  $\mu\text{m}$  in (C)–(G). bra, brachiolar apparatus; pl, primary larval body; pj, primary juvenile (adult) rudiment; rsj, rudiment of secondary juvenile (adult); sl, secondary larval body; sj, secondary juvenile (adult).

those we observed in regenerating asteroid larvae, with aggregations of mesenchyme cells with pseudopodia evident at the site of bisection (Vickery, 2002). Elongation of the larval skeleton resulted in replacement of the larval arms (Fig. 9E). Eight days after bisection, both larval portions had completely regenerated missing larval structures (Fig. 9F, G). Two weeks after bisection, regenerated larvae were morphologically and functionally indistinguishable from non-bisected control larvae. They displayed normal swimming and feeding behaviors. Eighteen days after bisection, regenerated larvae developed adult rudiments on the posterior and metamorphosed into normal juveniles.

In larvae of the irregular echinoid *D. excentricus*, the regeneration processes were almost identical to those in larvae of *L. variegatus*. Six days after bisection, the digestive system of anterior portions of larvae had almost completed regeneration, although the anus had yet to form (Fig. 10A, B). Elongation of the larval skeleton was observed in the regenerating posterior larval portion, while the exposed larval skeleton was surrounded by newly regenerated epidermis and ciliary bands (Fig. 10A, C). Although posterior portions of larvae were capable of complete regeneration, the anterior portions regenerated only partially during the course of the study (about 2 weeks).



**Figure 6.** Two weeks after surgical removal of the far anterior tips of the bipinnariae of *Pisaster ochraceus*, the anterior tips showed no signs of regeneration of the larval body but continued to actively swim, while the posterior portions had partially regenerated the missing tips. (A) Anterior tip (including axocoel) immediately after bisection. (B) Anterior tip 2 weeks after bisection. (C) Posterior portion approximately 1 week after bisection. Scale bars = 50  $\mu\text{m}$  in (A)–(B), 200  $\mu\text{m}$  in (C).

### Discussion

The present study demonstrates that planktotrophic larvae of the asteroid *Luidia foliolata* and *Pisaster ochraceus* possess extensive regenerative capacities regardless of their developmental stage. In early and mid-stage surgically bisected larvae, both species regenerated missing body components within 2 weeks; and no mortality occurred due to bisection, a result similar to our previous observations (Vickery and McClintock, 1998). Mesenchyme cells possessing pseudopodia played a regenerative role in the formation of an entirely new digestive system. In early-stage larvae, a new pair of coelomic pouches was formed by evagination of the anterior portion of the newly formed digestive system in association with aggregations of mesenchyme cells (possessing pseudopodia). A similar type of coelom formation was documented in echinoid larvae that had been surgically manipulated (reviewed in Hörstadius, 1973, and Vickery *et al.*, 2001b). The resultant coelomic

structures are essential body components that eventually form the body cavity in adult organisms, ultimately surrounding the digestive and reproductive organs. Coelomocytes (located within the coelom) have been reported to participate in regenerative processes in adult echinoderms (Thorndyke *et al.*, 1999). In contrast, we found that bisected larvae, lacking coelomic pouches in their anterior regions, completed regeneration. Thus, coelomocytes apparently do not play a critical role in larval regeneration.

In adult asteroids, regeneration is initiated by a proliferation of epidermal cells forming a mesenchymatous blastema, which later gives rise to the missing body structures (Candia Carnevali and Bonasoro, 1994; Bonasoro *et al.*, 1998; Thorndyke *et al.*, 1999). Our observations demonstrate that regenerative processes occur in a similar fashion in planktotrophic asteroid larvae. Our microscopic exami-



**Figure 7.** No regeneration of the larval body from the surgically removed brachiolar apparatus was observed in *Pisaster ochraceus*, but the larval posterior portion regenerated the brachiolar apparatus within 7 days, and within 2 weeks the larva was morphologically and functionally indistinguishable from non-bisected control larvae. (A) A swimming brachiolar apparatus with axocoel immediately after surgical removal. (B) A swimming brachiolar apparatus (lateral view) 2 weeks after surgical removal. Morphological changes are evident. (C) Larval posterior portion with regenerating brachiolar apparatus (arrow) about 5 days after surgical bisection. Scale bars = 50  $\mu\text{m}$  in (A)–(B), 200  $\mu\text{m}$  in (C).





**Figure 8.** When posterolateral arms were removed from *Pisaster ochraceus* brachiolaria larvae, the formation of (arm) buds was often observed at the site of amputation, while the severed arms continued to swim (with no sign of regeneration) for over 2 weeks. In most cases, regenerated posterolateral arms never reached the same length as before. (A) Regenerating (arm) bud (arrow). (B) Severed posterolateral arm immediately after bisection. After 2 weeks the severed arm still continued to actively swim (C), and muscle contractions were evident (D). Scale bars = 200  $\mu\text{m}$  in (A), 50  $\mu\text{m}$  in (B)–(D).

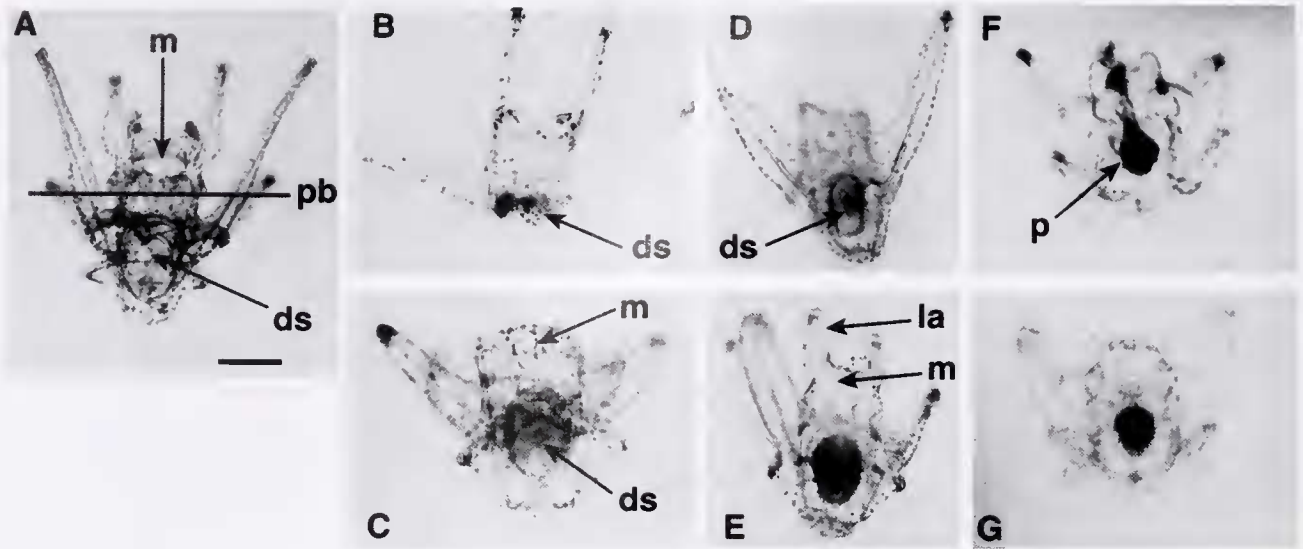
nations revealed that a proliferation of cells was initiated at the larval epithelium (ectoderm) near the surgical plane. During the process of regeneration, these cells appeared to dedifferentiate into mesenchymal-like cells and then to redifferentiate into new types of cells that later gave rise to the regenerated structures (Thorndyke *et al.*, 1999). However, further confirmation is necessary.

Cell fate in echinoderm larvae has previously been considered by some researchers to be determinate and irreversible once embryos or larvae attain discrete developmental stages (Cameron *et al.*, 1987; Cameron and Davidson, 1991; Davidson *et al.*, 1995; Thorndyke *et al.*, 1999). Nonetheless, the flexibility of developmental pathways and morphogenesis has been questioned lately (Balser, 1998). Our results demonstrate that fully differentiated larval body tissues are capable of additional proliferation and apparent dedifferentiation and redifferentiation in order to reconstruct missing

body parts, including the larval epidermis and coelomic pouches, suggesting organogenesis from previously differentiated cells. Moreover, our observations are supported by those of Balser (1998) in asexually cloning ophiuroid larvae, in which cloning apparently required both development and growth of new tissues as well as a reorganization of some existing tissues. Thus, many cells apparently remain omnipotent or pluripotent (capable of differentiation into any type of cell) in the larval stages, at least in the planktotrophic echinoderm species examined to date. However, since our studies and those of Balser (1998) have primarily relied upon morphological observations, future work using cell marking or other methods to follow the fate of individual larval cells is required to absolutely determine whether dedifferentiation/redifferentiation and organogenesis from previously differentiated cells occurs during larval regeneration.

Peterson *et al.* (1997), discussing larval forms (including planktotrophic echinoderm larvae) with what they termed "maximal indirect development" (Davidson *et al.*, 1995), suggested that larva-specific embryonic cell lineages with a fixed fate and a limited capacity for division give rise to the larval structures, whereas pluripotent "set-aside" cells, capable of relatively unlimited cell division, give rise to the adult form. The results of the present study and others (Jaecle, 1994; Balser, 1998; Vickery and McClintock, 1998) suggest that in planktotrophic echinoderm larvae the fate of larval-specific cells is not fixed, and their division capacity is not limited to only a few division cycles as was suggested by Peterson *et al.* (1997). Since it has been demonstrated that omnipotent or pluripotent larval cells, which remain versatile even after differentiation into specific larval tissues, are capable of replacing missing larval structures, it is questionable whether pluripotent set-aside cells are required to give rise to the adult structures as was suggested by Peterson *et al.* (1997). Further studies should shed more light on this subject.

In both asteroid species we examined, the anterior portions of bisected larvae lost their functional gut as a result of surgical bisection, retaining only the upper part of the mouth and oral cavity. Therefore, these larval anterior portions could not feed on phytoplankton until they had regenerated a functional digestive system; they decreased in size for 7 days until the digestive system had regenerated, restoring their capacity to feed. It is possible that they obtained some nutrients directly through the larval epidermis (ectoderm) in the form of dissolved organic matter from the surrounding seawater (Stephens, 1972, 1981; Manahan, 1990; Jaecle and Manahan, 1989, 1992). Also, the bisected, regenerating anterior portions apparently reabsorbed some larval body parts as a nutrient source during regeneration of the digestive system (this would partly explain their decrease in body size). Absorption and utilization of the larval body during regeneration was suggested by Chia and Burke (1978). In

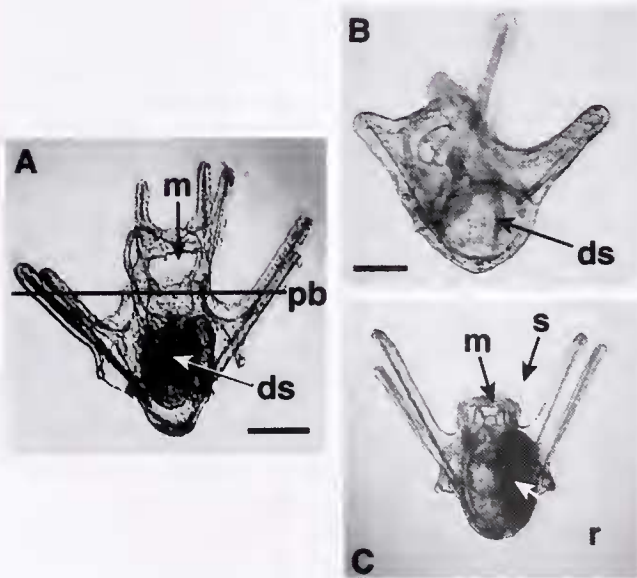


**Figure 9.** Surgically bisected pluteus larva of the regular echinoid *Lytechinus variegatus*. (A) Non-bisected echinopluteus larva. (B) Regenerating anterior portion of larva immediately after bisection. (C) Regenerating posterior portion of larva immediately after bisection. (D) Regenerating anterior portion of larva 5 days after bisection. (E) Regenerating posterior portion of larva 5 days after bisection. Missing structures from both the anterior and posterior larval halves (including the larval digestive system) are seen undergoing regeneration. (F) Regenerated anterior portion of larva 8 days after bisection. (G) Regenerated posterior portion of larva eight days after bisection. ds, digestive system; la, larval arm; m, mouth; p, phytoplankton; pb, plane of bisection. Scale bars = 200  $\mu\text{m}$  (A), and 100  $\mu\text{m}$  (B-G, shown in B).

contrast, the posterior portions of bisected larvae, which retained functional digestive systems from the esophagus to the anus (and therefore continued to actively feed) increased

in size over a 2-week period. Both anterior and posterior portions of bisected larvae of both species somehow obtained the required energy or nutrition to support regeneration of their missing structures, either through active feeding, absorption of dissolved organic matter, or absorption of larval body tissues. Future investigations are necessary to evaluate the energetics of the regeneration process in planktotrophic asteroid larvae. In the present study we used size (larval length) as an indicator for growth during regeneration. In future studies it also would be valuable to examine larval body mass, the thickness of larval tissues, or both in addition to body length during regeneration at various larval stages. Under certain conditions tissues could become thinner to accommodate an overall increase in body length.

In late-stage larvae, the anterior portions of both species regenerated in a process identical to that described for the early and mid-stage larvae. The posterior portions (with adult rudiment) of *L. foliolata*, however, instead of regenerating their missing anterior larval components, rapidly completed metamorphosis into juvenile asteroids within 3 days of bisection (Vickery and McClintock, 1998). Apparently surgical bisection either initiated or dramatically accelerated the process of metamorphosis. This suggests that incorporation of the entire larval body mass into the juvenile is not required during the metamorphic process, contradicting the report of Chia and Burke (1978) for larvae of the sand dollar *Dendraster excentricus*. Future studies examining the energetics of larval regeneration and metamorpho-



**Figure 10.** Surgically bisected pluteus larva of the irregular echinoid *Dendraster excentricus*. (A) Non-bisected echinopluteus larva. (B) Regenerating anterior portion of larva 6 days after bisection. (C) Regenerating posterior portion of larva 6 days after bisection. ds, digestive system; m, mouth; r, rudiment; pb, plane of bisection. Scale bars = 200  $\mu\text{m}$  (A), and 100  $\mu\text{m}$  (B-C, shown in B).

sis, and comparing the internal anatomy and survival and fate of juveniles resulting from bisected larvae to the internal anatomy and survival and fate of juveniles resulting from non-bisected controls, will be of great interest.

In contrast, the posterior portions of *P. ochraceus* larvae (which lacked the brachiolar apparatus) did not readily complete metamorphosis following surgical bisection. Instead they regenerated their missing larval anterior components, including the brachiolar apparatus, prior to metamorphosis. The brachiolar apparatus is considered to be important for metamorphosis (Baker, 1978). These observations are probably due to developmental differences (described below) that characterize *L. foliolata* and *P. ochraceus*. *L. foliolata* is a member of the family Luidiidae, which lacks a brachiolaria larval stage in its ontogeny (Chia *et al.*, 1993). These larvae complete metamorphosis while swimming in the water column (Chia *et al.*, 1993). In contrast, *P. ochraceus* is a member of Asteroiidae family and possesses a brachiolaria larval stage. Larvae of this species require attachment of the brachiolar apparatus to a favorable substrata in order to complete metamorphosis (Baker, 1978; Chia *et al.*, 1993). Therefore, it is possible that metamorphically competent posterior portions of bisected larvae of *L. foliolata*, which do not require a brachiolar apparatus for substrata attachment to trigger metamorphosis, rapidly complete metamorphosis following surgical bisection to avoid unnecessary feeding and growth to reform anterior tissues that are not required for metamorphosis. The remaining portion of the larval body is absorbed into the adult rudiment during metamorphosis. *P. ochraceus*, on the other hand, requires the brachiolar apparatus for attachment and metamorphosis; thus it is reasonable that this structure would have to be regenerated before metamorphosis.

A number of theories about the evolution of developmental modes in asteroids and marine invertebrate larvae in general have been proposed (reviewed by McEdward and Janies, 1993; Wray and Raff, 1991). Within planktotrophic modes of development in Asteroidea, the Luidiidae are often considered to possess more primitive development than the Asteroiidae (Downey, 1973; Clark and Downey, 1992; McEdward and Janies, 1993). Others argue exactly the opposite (Blake, 1988). Our observations do not clearly support either hypothesis; however, they do demonstrate that a brachiolar apparatus is necessary for development in the Asteroiidae and that there are clearly regenerative differences between Luidiidae and Asteroiidae.

When small portions of the larval body were removed from *P. ochraceus*, the missing portion of the larval body usually regenerated. Some of these surgical procedures produced anterior larval body fragments almost identical to those Jaeckle (1994) observed in larva from plankton tow samples. Jaeckle reported that these fragments, which were produced by autotomization of the far anterior portion of the larval preoral lobe, developed into

bipinnaria larvae in 1–2 days. We did not consistently observe regeneration of the entire larval body from our surgically removed anterior portions of the preoral lobe over the 2 weeks they were followed. However, Jaeckle's larval fragments were maintained at higher seawater temperatures than ours, and their developmental processes might have been accelerated.

One exception to this pattern was in brachiolaria larvae that had lost their larval arms. In mid-stage larvae, missing portions were regenerated even as development progressed (the adult rudiment was forming). No mortality of small larval fragments was observed during the 2-week studies. Subsequent observations indicated that these small larval pieces continued to swim in the water column for up to 4 weeks, suggesting they may obtain energy or nutrients for maintenance through the uptake of dissolved organic matter from the surrounding seawater (Stephens, 1972, 1981; Manahan, 1990; Jaeckle and Manahan, 1989, 1992).

Our studies further demonstrate that the planktotrophic larvae of regular and irregular echinoids are capable of complete regeneration following surgical bisection. Additional studies are needed to determine whether bisected anterior portions of larvae of *D. excentricus* can fully regenerate and metamorphose into juveniles. The inability of the anterior larval portion of *D. excentricus* to fully regenerate over a 2-week period may be related to the comparatively low ambient seawater temperatures at which these larvae were reared (12–15 °C for *D. excentricus* compared to 20–23 °C for *L. variegatus*). Lower temperature can suppress metabolic rates in developing marine invertebrate larvae (Pearse *et al.*, 1991; Boidron-Metairon, 1995; Marsh *et al.*, 2001).

The planktotrophic larvae of asteroids are morphologically similar to those of holothuroids (Smiley, 1986), whereas the planktotrophic larvae of echinoids are similar to those of ophiuroids (Levin and Bridges, 1995). Ophiuroid larvae, like asteroid larvae, undergo asexual clonal reproduction (involving regeneration of a complete new larval body) (Balsler, 1998). Therefore, it is likely that planktotrophic larvae of most if not all echinoderms have the capacity for regeneration. This capacity may help offset their presumably high mortality rates in the plankton (Menge, 1975; Vickery and McClintock, 1998).

Research in the area of regeneration of echinoderm larvae has only begun (Balsler, 1998; Vickery and McClintock, 1998; Vickery, 2001, 2002; Vickery *et al.*, 2001a, 2001b). Because echinoderms share many developmental traits with other deuterostomes, an understanding of the processes of regeneration in echinoderm larvae may lead to a better understanding of regeneration in many higher animals, including vertebrates.

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